P. ENT COOPERATION TREA

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 02 November 2000 (02.11.00)	
International application No. PCT/EP00/02508	**Applicant's or agent's file reference** 5254/00/WO-Koe
International filing date (day/month/year) 22 March 2000 (22.03.00)	**Priority date** (day/month/year) 25 March 1999 (25.03.99)
Applicant BIRYUKOV, Sergey Vladimirovich et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

12 August 2000 (12.08.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Manu Berrod
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

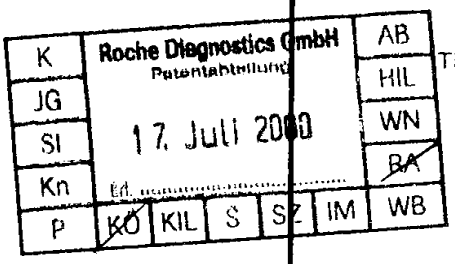
From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
ROCHE DIAGNOSTICS GMBH
- Patentabteilung -
D-68298 Mannheim
GERMANY



Applicant's or agent's file reference: 5254/00/WO-Koe	Date of mailing (day/month/year): 14/07/2000
	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No.: PCT/EP 00/ 02508	International filing date (day/month/year): 22/03/2000
Applicant: ROCHE DIAGNOSTICS GMBH et al	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Mireille Claudepierre

Form PCT/ISA/220 (July 1998)

T: 14.09.00 cov. Se

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When? Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged,

(ii) the claim is cancelled,

(iii) the claim is new,

(iv) the claim replaces one or more claims as filed,

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
5254/00/WO-Koe		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 00/ 02508	22/03/2000	25/03/1999
Applicant		
ROCHE DIAGNOSTICS GMBH et al		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of Invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 3

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [X] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/02508

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P21/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, CAB Data, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93 07287 A (PROMEGA CORP) 15 April 1993 (1993-04-15) the whole document ---	
A	WO 94 18371 A (SEMER S A ;DALLA VALLE FLORISVALDO (BR)) 18 August 1994 (1994-08-18) the whole document ---	
A	US 5 593 856 A (CHOI CHA-YONG ET AL) 14 January 1997 (1997-01-14) cited in the application the whole document --- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 July 2000	14/07/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hornig, H

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KIM D M ET AL: "A semicontinuous prokaryotic coupled transcription/translation system using a dialysis membrane" BIOTECHNOLOGY PROGRESS,XX,XX, vol. 12, no. 5, September 1996 (1996-09), pages 645-649-649, XP002106860 ISSN: 8756-7938 cited in the application the whole document	
A	US 5 478 730 A (ALAKHOV JULY B ET AL) 26 December 1995 (1995-12-26) cited in the application the whole document	
P,A	WO 99 50436 A (ROCHE DIAGNOSTICS GMBH ;BIRYUKOV SERGEY VLADIMIROVICH (RU); MAJORO) 7 October 1999 (1999-10-07) the whole document	

INTERNATIONAL SEARCH REPORT

[redacted]ormation on patent family members

[redacted]nternational Application No
PCT/EP 00/02508

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9307287 A	15-04-1993	AT 147104 T	15-01-1997
		AU 660329 B	22-06-1995
		AU 2792192 A	03-05-1993
		DE 69216385 D	13-02-1997
		DE 69216385 T	12-06-1997
		DK 566714 T	16-06-1997
		EP 0566714 A	27-10-1993
		ES 2097363 T	01-04-1997
		GR 3022719 T	30-06-1997
		JP 2904583 B	14-06-1999
		JP 6503477 T	21-04-1994
		US 5324637 A	28-06-1994
		US 5665563 A	09-09-1997
WO 9418371 A	18-08-1994	BR 9300343 A	27-09-1994
US 5593856 A	14-01-1997	KR 131166 B	11-04-1998
		JP 7298893 A	14-11-1995
US 5478730 A	26-12-1995	SU 1705302 A	15-01-1992
		AT 123308 T	15-06-1995
		CA 1318626 A	01-06-1993
		CN 1043743 A	11-07-1990
		DD 279270 A	30-05-1990
		DE 58909270 D	06-07-1995
		EP 0401369 A	12-12-1990
		JP 3503479 T	08-08-1991
		WO 9007003 A	28-06-1990
		AT 130633 T	15-12-1995
		AT 147787 T	15-02-1997
		CA 2064685 A,C	01-02-1991
		CA 2064754 A,C	01-02-1991
		DE 69023773 D	04-01-1996
		DE 69023773 T	24-10-1996
		DE 69029744 D	27-02-1997
		DE 69029744 T	10-07-1997
		DK 593757 T	07-07-1997
		EP 0485608 A	20-05-1992
		EP 0593757 A	27-04-1994
		JP 2891540 B	17-05-1999
		JP 5505095 T	05-08-1993
		WO 9102075 A	21-02-1991
		WO 9102076 A	21-02-1991
WO 9950436 A	07-10-1999	NONE	

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

ROCHE DIAGNOSTICS GMBH
- Patentabteilung -
D-68298 Mannheim
ALLEMAGNE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)* 08.12.2000

Applicant's or agent's file reference
5254/00/WO-Koe

IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/02508	22/03/2000	25/03/1999

Applicant
ROCHE DIAGNOSTICS GMBH et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/

European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer

Gallego, A

Tel.+49 89 2399-8102

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

4

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
5254/00/WO-Koe		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/02508	22/03/2000	25/03/1999
International Patent Classification (IPC) or national classification and IPC C12P21/02		
Applicant ROCHE DIAGNOSTICS GMBH et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
12/08/2000	08.12.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Döpfer, K-P Telephone No. +49 89 2399 8547

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/02508

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-26 as originally filed

Claims, No.:

1-9 as originally filed

Drawings, sheets:

1/7-7/7 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-9
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-9
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-9
	No:	Claims	

2. Citations and explanations
see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item I

Basis of the report

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents

   D1: WO 93 07287 A (PROMEGA CORP) 15 April 1993 (1993-04-15)
   D2: WO 94 18371 A (SEMER S A ;DALLA VALLE FLORISVALDO (BR)) 18 August 1994 (1994-08-18)
   D3: US-A-5 593 856 (CHOI CHA-YONG ET AL) 14 January 1997 (1997-01-14) cited in the application
   D4: US-A-5 478 730 (ALAKHOV JULY B ET AL) 26 December 1995 (1995-12-26) cited in the application
   D5: WO 99 50436 A (ROCHE DIAGNOSTICS GMBH ;BIRYUKOV SERGEY VLADIMIROVICH (RU); MAJORO) 7 October 1999 (1999-10-07) (does not belong to the prior art according to Rule 64 PCT).
   D6: KIM D M ET AL: 'A semicontinuous prokaryotic coupled transcription/translation system using a dialysis membrane' BIOTECHNOLOGY PROGRESS,XX,XX, vol. 12, no. 5, September 1996 (1996-09), pages 645-649-649, XP002106860 ISSN: 8756-7938 cited in the application

2. The present application relates to a method for obtaining polypeptides in a cell-free system characterised by the choice of the parameters, mode of synthesis, type and parameters of at least one porous barrier. The concentrations of the selected components determining the productivity of the process are changed between the upper and lower limits as defined.

3. *Novelty and Inventive Step (Article 33(2)(3) PCT)*

   The cited prior art documents disclose methods for the cell-free preparation of

peptides/proteins.
D1 deals with the variation of concentration of Mg^{2+} in order to increase the protein yield.
D2 is considered not relevant for the assessment of novelty and inventive step of the subject-matter of the present application.
D3 and D4 address the use of porous barriers in the production of peptides or proteins by means of cell-free systems.
D6 describes a cell-free protein synthesis system (coupled transcription/ translation reaction) using a dialysis membrane. This paper discloses also the influence of the supplementation of certain essential components on the final protein yield (cf Table 1).

However, none of the prior art documents mentions the controlled shift of the concentration of essential components between (predefined) upper and lower limits as a technical feature of the process. Thus, the subject-matter of present claims 1-9 is regarded as novel.

D4 is considered representing the closest prior art. Neither the closest prior art document alone nor in combination with the other documents cited in the International search suggest the controlled shift between the upper and lower limit of a predefined concentration range of a selected component. The improvement in the productivity obtained by this measure as shown in Figs. 10 to 13 could not be expected from the knowledge of the prior art teachings. Thus, inventive step can be acknowledged for present claims 1-9.

4. *Industrial applicability (Article 33(4) PCT*

The subject-matter of present claims 1-9 appear to comply with the requirements of industrial applicability as stipulated in Article 33(4) PCT.

Re Item VI

Certain documents cited

Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
WO-A-99/50436	07.10.1999	27.03.1999	31.03.1998

Re Item VII

Certain defects in the international application

1. The title of the reference DE Patent Appl. 19832160.0 A1 should read "Verfahren und Vorrichtung zur Durchführung biochemischer Reaktionen" (page 29, line 17).

Re Item VIII

Certain observations on the international application

1. Although the acronym NTP, which obviously means nucleoside triphosphate, is known to the skilled person, it should be explained at least once in the description in order to meet the requirements of clarity (cf claims, Article 6 PCT).

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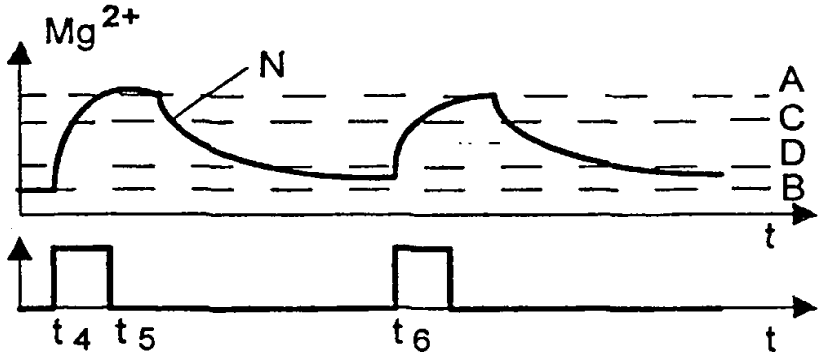
(54) Title: METHOD FOR SYNTHESIS OF POLYPEPTIDES IN CELL–FREE SYSTEMS

(57) Abstract

The method of polypeptide synthesis in eukaryotic or porkaryotic cell–free systems based on a modified version of synthesis in the continuous flow or continuous exchange modes when, in addition to input into the reaction mixture of components maintaining the synthesis and removal from the reaction mixture of low molecular weight components inhibiting the synthesis, the concentration of at least one of the components selected from the group consisting of Mg^{2+}, K^+, NTP, polyamines or their combinations determining the productivity of the synthesis is continuously changed within the given range of concentrations, while the concentrations of the other components are maintained constant.



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METHOD FOR SYNTHESIS OF POLYPEPTIDES IN CELL-FREE SYSTEMS

FIELD OF THE INVENTION

The invention pertains to the field of molecular biology, in particular to synthesis of proteins and polypeptides in cell-free systems prepared from prokaryotic and eukaryotic cells.

BACKGROUND

Synthesis of polypeptides and proteins in cell-free translation systems of the first generation (US Patent 4668624, Roberts, 1979) was performed in a static (batch) mode where the reaction mixture is in static conditions with constant Mg^{2+}, K^{+} and NTP concentrations, constant pH and temperature. To this end, extracts and lysates of prokaryotic (Zubay, 1973) and eukaryotic cells (Roberts and Paterson, 1973; Pelham and Jackson, 1976) were prepared, and natural and synthesized mRNAs were used (US Patent 4937190, Palmberg, 1990).

Rapid development of biotechnology has called for alternative methods that would increase the yield of synthesized proteins. The design of more productive translation systems in which the concentration of basic components is maintained constant during the synthesis is one direction of efforts aimed at improvement of the existing methods. In the second generation systems (Spirin et al., 1988), a continuous flow of low weight substrates included in the feeding solution (CFCF mode) into the reactor volume and removal of target polypetides and low molecular weight products inhibiting the cell-free system increases the time of its operation and raises the yield of the desired protein as compared to the classic system of synthesis in static (batch) conditions. Numerous studies have been focused on optimization of the conditions for CFCF protein synthesis (Baranov, 1989; Ryabova et al., 1989; Takanori et al., 1991; Spirin, 1992, Baranov and Spirin, 1993; Volyanik et al., 1993; Erdman et al.,1994; Kim and Choi, 1996; Yamamoto, 1996; Ryabova et al., 1998, EP Patent 0312617; Alakhov et al., 1993, EP Patent 0401369, Baranov et al., 1995, US Patent 5434079; Mozayeni, 1995; JP Patent 7075592, Shimizu, 1995; JP Patent 7031494, Sakurai, 1995; JP Patent 5076381, Sato, 1995; EP Patent 0593757, Baranov et al., 1997; US Patent 5593856, Choi et al., 1997).

US Patent 5478730 (Alakhov et al., 1995) describes a method in which the synthesis in cell-free translation systems is based on continuous exchange (CECF mode) of the feeding solution components with the component of the reaction mixture via a semipermeable barrier by a diffusion process. The results obtained by many authors (Davis et al., 1996; Kim and Choi, 1996; US Patent 5593856, Choi, 1997; JP Patent 10080295, Yamane 1998)

demonstrate a significant increase in the yield of the target polypeptide upon continuous exchange, as compared to the static (batch) mode of operation.

In addition to improvement of the components of the translation system, efforts were made to improve methods for preparation of mRNA in transcription systems including RNA polymerase and DNA. In these systems, preparation of mRNA depends on the concentration of RNA polymerase and DNA, as well as on the concentration of Mg^{2+}, K^+ and NTP and other ionic conditions (Kern and Davis, 1997). The cost of components of the *in vitro* transcription including RNA polymerase, DNA and NTP is very high. Therefore it is necessary to analyze conditions of transcription and optimize the process of mRNA preparation (Gurevich et al., 1991).

There are methods for synthesis of polypeptides in a CFCF mode in prokaryotic cell-free systems in conditions of a coupled transcription-translation (Baranov et al., 1989; EP Patent 0401369, Baranov et al., 1995; Ryabova et al., 1998) and the process was patented where transcription and translation occur in eukaryotic cell-free systems in the same reaction volume (Spirin, 1992; Baranov and Spirin, 1993; EP Patent 0593757, Baranov et al., 1997).

It is known (Craig et al., 1993) that translation and transcription conditions in eukaryotic cell-free systems differ and are determined largely by the concentrations of Mg^{2+} and K^+. Therefore, two-stage (US Patent 5665563, Beckler, 1997; Operating Guide, Single Tube Protein™, Novagen Inc., 1998) or three-stage synthesis (Roberts and Paterson, 1973) is widely used in a static (batch) mode. At the first stage optimal conditions are achieved for mRNA transcription, then the mRNA is purified or immediately added to a new reaction mixture with conditions for translation. A one-stage synthesis of polypeptides in a transcription-translation eukaryotic cell-free systems is known (US Patent 534637, Thompson et al., 1994; Operation Guide, Linked in vitro SP6/T7 Transcription/Translation Kit, Roche Diagnostics GmbH, 1998). The authors of the patent (US Patent 5324637, Thompson et al., 1994) used a known principle of optimization of Mg^{2+} concentration in the reaction mixture. By adding Mg^{2+} to the reaction mixture prior to the synthesis, they achieved such a concentration of Mg^{2+} in the reaction system which is intermediate between the transcription optimum and the translation optimum. Further studies showed that such optimization has no advantages over the two-stage or three-stage procedures. The study of Laios et al. (1998) demonstrates that optimization of separate stages of transcription and translation is from 2 to 6 times more efficient than that of a coupled process. On the other hand, optimization of the selection of Mg^{2+} concentrations is based on a preliminary measurement of the magnesium concentration in the lysate or in the reaction volume which devalues the principle of the one-stage procedure.

European Patent 0593 757 (Baranov et al., 1997) describes the possibility to perform continuous CFCF synthesis of polypeptides in eukaryotic cell-free transcription-translation systems for 20 hours. During the synthesis, the Mg^{2+} concentration in the reaction mixture is maintained at the required level due to the constant concentration of Mg^{2+} in the feeding solution. Since ribonuclease activity in the reaction volume is low and the mRNA templates retain their activity for a prolonged time, the reaction system works with both the earlier and newly synthesized mRNA templates and synthesizes a target product due to the constant Mg^{2+} concentration. For a more productive synthesis, the transcription system should synthesize an adequate amount of mRNA. Therefore a large quantity of expensive polymerase SP6 or T7 (30,000 units) is required. It is mentioned in the text of the patent that optimal conditions of synthesis should be chosen in each individual case. To make an appropriate choice, it is necessary to perform a series of syntheses in a batch volume at different Mg^{2+} concentrations and determine its optimal value for the given polypeptide. Optimization of the process is time consuming and rather expensive.

There are many devices in which the continuous exchange mode (CECF) is maintained due to a diffusion process. The device, in the form of a dialysis container for synthesis of polypeptides in a cell-free system, was first described in US Patent 5478730 (Alakhov et al., 1995). Promega Corp. (Davis et al., 1996) made a comparative analysis of syntheses (in a static (batch) mode and at a continuous exchange mode) during coupled transcription-translation in a *E. coli* prokaryotic cell-free system. To this end, the authors used "DispoDialyser" instruments manufactured by Spectrum Medical Industr. (US Patent 5324428, Flaherty, 1994) and "Slidealyzer" dialysers manufactured by Pierce Chemical Comp. (US Patent 5503741, Clark, 1996).

For the synthesis of polypeptides upon coupled transcription-translation in a preliminarily concentrated prokaryotic cell-free system of *E. coli*, Kim and Choi (1996) used a dialysis membrane fixed at the bottom of a cylinder.

Yamamoto (1996) constructed a dialyser in which the membrane is made from hollow fibers. The feeding solution passes through the hollow fibers. Due to diffusion, the components of the reaction mixture exchange with those of the feeding solution.

In the device designed by Yamane (JP Patent 100809295, Yamane, 1998), the membrane is used to maintain constant conditions of synthesis due to diffusion of low molecular substrates of the feeding solution circulating along the dialysis membrane.

US Patent 5478730 (Alakhov et al., 1995) is most close to the dialyser operating in a continuous exchange mode. The authors of the patent give a detailed description of

requirements for the porous barrier made either of a dialysis membrane, flat membrane or hollow fibers, which can be composed in multi-layered structures.

Many devices whose operation is based on a continuous flow (CFCF) mode have been developed. They differ from each other by the formation of the feeding solution flows and the modes of removal of products of synthesis and metabolism inhibiting operation of the system.

The use of one ultrafiltration membrane in a flow-type reactor is described in many papers (Spirin et al., 1988, 1992; Takanori et al., 1991; Spirin, 1992; Volyanik et al., 1993; Kim and Choi, 1996; Ryabova et al., 1998). A disadvantage of this method is that the incoming flow of the feeding solution is equal to the volume of the outcoming flow of low molecular and high molecular components resulting in fast closing of the pores of the ultrafiltration membrane.

In 1990 Fischer et al. (DE Patent 3914956) proposed a method using a multifold pulse supply of the feeding solution to the reaction volume. To this end, N cycles are formed to provide positive and negative pressure in the reaction volume. Upon formation of positive pressure, the inhibiting products are removed from the reaction volume via the porous barrier and mixed with the feeding solution. At negative pressure, part of the inhibiting products are returned to the reaction volume via the porous barrier together with another portion of the feeding solution. Moreover, high molecular weight components of the cell-free system required for a prolonged synthesis are intensely washed out from the reaction mixture.

In 1995 Mozayeny (US Patent 5434079) proposed a device with improved removal of high molecular weight products due to an increased area of the ultrafiltration membrane. During synthesis the components of the cell-free system are removed together with the target product via the large area of two parallel membranes with pore sizes from 70 to 100 kD, which limits the time of the synthesis.

The devices proposed herein are most close to the device described in US Patent 5478730 (Alakhov et al., 1995) with one or two porous barriers. The barriers can be made of flat membranes or hollow fibers.

Generally, the prior art describes methods and devices developed for maintaining constant conditions during the synthesis. Constant conditions are provided both by removal from the reaction volume the low molecular weight products which inhibit operation of the cell-free system and by supplying into the reaction volume some components which maintain the synthesis. The synthesis is maintained by the same concentrations of Mg^{2+}, K^{+} and NTP and other components both in the reaction mixture and in the feeding solution. The authors of the prior art patent use a well known principle of optimization. Optimization of the process is time consuming and rather expensive.

SUMMARY OF THE INVENTION

The object of the present invention is to provide a method that will allow the synthesis of a target polypeptide in prokaryotic and eukaryotic cell-free systems. The invention is based on modification of methods of synthesis in a continuous flow (CFCF) mode or a continuous exchange (CECF) mode. In these modes, during the synthesis parallel to input into the reaction mixture of components maintaining the synthesis and output from the reaction mixture of low molecular weight components inhibiting the synthesis, concentrations of at least one of the selected components determining the productivity of the synthesis (Mg^{2+}, K^+, NTP, polyamines or their combinations) are continuously changed from the upper to the lower limit of the determined range.

LIST OF FIGURES

The invention is illustrated by the following figures.

Figure 1 shows diagrams of changes in Mg^{2+} concentrations upon synthesis of mRNA and synthesis of polypeptides in a cell-free system operating in a continuous exchange mode (CECF).

Figure 2 shows a diagram of changes of Mg^{2+} concentrations upon synthesis in a continuous flow mode (CFCF) when the conditions of synthesis are changed from predominantly transcriptional to predominantly translational.

Figure 3 shows a diagram of changes in Mg^{2+} concentrations at a recurrent pulse input of the additional mixture to the reaction mixture.

Figure 4 demonstrates a diagram of recurrent changes in Mg^{2+} concentrations according to the linear gradient shape.

Figure 5 shows a scheme of a reactor with one porous barrier.

Figure 6 shows a scheme of a reactor module and directions of flows formed in the mode of branched output of high molecular weight and low molecular weight fractions (CFCF-BF).

Figure 7 represents a scheme of branched flows when the target product is removed from the zone of synthesis (CFCF-RP).

Figure 8 shows a scheme of a reactor module and flow branching when the first porous barrier plays the role of a distributor of flows of the feeding solution and the additional mixture with the target product remaining in the zone of synthesis (CFCF-RP).

Figure 9 represents a scheme of a reactor module and flow branching when directions of the feeding solution input are recurrently switched from the first porous barrier to the second one (CFCF-RF).

Figure 10 shows the kinetics of CAT synthesis. Diagram P refers to the synthesis in a static (batch) mode. Diagram R demonstrates the kinetics of CAT synthesis upon translation in the CECF mode.

Figure 11 shows the kinetics of CAT synthesis in the combined transcription-translation system. Diagram S represents synthesis in the static (batch) mode. Diagram T represents the kinetics of CAT synthesis upon transcription-translation in the CECF mode.

Figure 12 shows the kinetics of CAT synthesis in the combined transcription-translation system with changing concentrations of Mg^{2+} and NTP in the reaction mixture during the synthesis. Diagram U represents synthesis in the (static) batch mode. Diagram V represents the kinetics of CAT synthesis upon transcription-translation in the CECF mode.

Figure 13 shows a diagram which compares the results of four experiments on the synthesis of the target CAT polypeptide.

DESIGNATIONS USED IN THE FIGURES:

F10, F11, F12 are the feeding solution flows.

F20, F21, F22 are the additional mixture flows.

F30, F31, F32 are flows of low molecular weight products of the reaction mixture.

F40 is the flow of high molecular weight products of the reaction mixture.

F50, F51, RF52 are flows of high molecular weight components maintaining the synthesis.

Positions 1 through 9 designate inlets and outlets of the reactor.

Positions 10 through 19 designate the reactor elements.

ABBREVIATIONS USED IN THE TEXT:

Mg^{2+}, magnesium ions added as a magnesium salt;

K^{+}, potassium ions added as a potassium salt.

DESCRIPTION OF SPECIFIC EMBODIMENTS

1. Stages of the synthesis

The synthesis consists of the following stages.

1. The reaction mixture is prepared using a cell lysate or cell extract.

2. The feeding solution and the additional mixture, which includes at least one of the selected components determining the productivity of the synthesis are prepared.
3. The mode of reactor operation is determined, the type of the reactor module is selected with a given number and types of porous barriers. The volume ratio of the reaction mixture and the feeding solution or the rate of the feeding solution flow via the reaction volume are determined.
4. The device for the synthesis including at least one reactor module is assembled.
5. The reaction mixture and the feeding solution are supplied to the corresponding zones of the reactor module separated by at least one porous barrier.
6. The additional mixture is supplied to the reaction mixture or to part of the feeding solution prior to or during the synthesis.
7. In the course of the synthesis, the additional mixture is introduced once, recurrently or continuously, depending on the mode of operation.
8. In the mode of preparative synthesis, the required high molecular weight components are supplied to the reaction mixture once, recurrently or continuously.
9. The synthesized product is collected from the reaction mixture either at the end of synthesis or during the synthesis.
10. When the product is collected during the synthesis, it is analyzed and the conditions determining the system productivity are corrected.

2. Preparation of reaction mixtures

Cell-free systems are prepared using cell lysates and cell extracts. They include all components necessary for protein synthesis as well as regeneration system, NTP, a buffer and salts, and amino acids.

A great variety of types of cell-free systems are known for synthesis of polypeptides (US Patent 5807717, Joyce, 1998). They are prepared from archeaebacteria (*Halobacterium, Thermoproteus, Methanococcus, Sulfolobus*, etc.), eubacteria (*Pseudonomas, Agrobacterium*, etc.) and eukaryotic cells (rabbit reticulocytes, wheat germ, HeLa, mouse liver, etc.). Conditions described herein are most close to those of translation systems and transcription-translation systems prepared from prokaryotic *E. coli* extracts (Zubay, 1973), eukaryotic wheat germ extracts (Roberts and Paterson, 1973) and extracts prepared from rabbit reticulocytes (Pelham and Jackson, 1976).

One of the components of the transcription-translation cell-free system should be a DNA-dependent RNA-polymerase that synthsizes mRNA. It is selected from *E. coli* RNA

polymerases or bacteriophage RNA polymerases. In this invention, we analyze but do not restrict to the use of polymerases T1, T3, T5, T7, SP6, A16, PHL1, PHL11 in such systems. The most suitable are polymerases T7 and SP6 polymerases.

3. Conditions of synthesis

It is known that in prokaryotic and eukaryotic cell-free systems conditions of synthesis differ. The productivity of synthesis depends on whether concentrations of such components of a cell-free system as Mg^{2+}, K^+ and NTP are within the optimal range and by what value they are changed during synthesis. The range of concentrations at which the synthesis is optimal is rather narrow. Any change in temperature, pH, initial concentrations of components during synthesis forces the cell-free system leave the mode of optimal synthesis, which results in a decreased yield of the synthesized product. For all cell-free systems, optimization means that the NTP value is a priori determined and such ranges of Mg^{2+} and K^+ concentrations are selected in which the synthesis is most productive.

This leads to a great scatter of "optimal" magnesium concentrations given in different patents. For example, as mentioned in Promega's patent (US Patent 5324637, Thompson et al., 1994), for polypeptide synthesis in a transcription-translation system with a reticulocyte lysate, optimal magnesium concentrations in the reaction mixture vary from 2.5 to 3.5 mM. However, as given in the other patent (US Patent 5807717, Joyce, 1998), this range is 6.0-10.0 mM Mg^{2+} for polypeptide synthesis in the same system with the reticulocyte lysate produced by Promega.

It is known (Pokrovskaya, 1994) that with the use of SP6, T7 and T3 polymerases optimal transcription takes place when Mg^{2+} concentration varied from 16 to 36 mM. Concentrations of NTP determine largely initiation of transcription (Guajardo et al., 1998), and Mg^{2+} concentrations should exceed the total concentrations of NTP (Gurevich et al., 1991; Kern and Davis, 1997) for an efficient action of the T7 polymerase. At the same time, for optimal translation of mRNA in cell-free systems with a reticulocyte lysate prepared by a standard technique (Pelham and Jackson, 1976; Suzuki, 1977; Merrick, 1983), magnesium concentrations may vary from 1.0 to 3.0 mM of Mg^{2+} added to the reaction mixture.

In this invention, contradictions appearing in determination of Mg^{2+} and K^+ concentrations, which provide the required level of both transcription and translation, are solved otherwise as compared to the known method when optimal concentrations of Mg^{2+} and K^+ are determined by results of intermediate experiments. Herein this is done in the following way. During the synthesis, parallel to input to the reaction mixture of components maintaining

the synthesis and output from the reaction mixture of low molecular weight components inhibiting the synthesis, concentrations of at least one of the selected components determining the productivity of the synthesis (Mg^{2+}, K^+, NTP, polyamines or their combinations) are continuously changed from the upper to the lower limit of the determined range.

The choice of the upper and lower limits of this range depends on the mode of synthesis, parameters of the cell-free extract, conditions of both the reaction mixture and the feeding solution. If Mg^{2+} is taken as one of the selected components, Mg^{2+} concentrations (for various modes including transcription, transcription-translation, translation) range from 0.25 to 50 mM of added Mg^{2+}. When one of the components of a cell-free system is DNA-dependent RNA polymerase, in the mode of RNA transription the upper and lower limits of a given range of changes in Mg^{2+} concentrations should be from 2 to 50 mM of added Mg^{2+}. If protein synthesis proceeds in conditions of transcription-translation, these limits should be from 2 to 25 mM of added Mg^{2+}. If protein synthesis proceeds in conditions of translation, the upper and lower limits of a certain range of changes in Mg^{2+} concentrations should be 0.25 and 25 mM of added Mg^{2+}, correspondingly. It is possible that these limits are chosen so that during synthesis, conditions of synthesis change together with the mode of synthesis (i.e. predominant transcription switches to transcription-translation or predominant translation). The previous example involves ranges of concentrations of only one of the selected components (Mg^{2+}) required for synthesis. The width of the selected ranges and their upper and lower limits are determined taking into account conditions of synthesis in prokaryotic and eukaryotic cell-free systems.

The other goal of this invention is to lower the cost of synthesis of a certain amount of polypeptide in eukaryotic cell-free systems. In the known methods, synthesis is performed at a high concentration of expensive T7 polymerase at a continuous flow via the reaction volume of expensive components of the feeding solution such as NTP and amino acids (European Patent 0593757, Baranov et al., 1997). In this invention, the productivity of synthesis in transcription-translation systems increases, since high concentrations of Mg^{2+} and NTP introduced to the reaction mixture at the beginning of synthesis decrease the amount of abortive mRNA, which in its turn reduces the expenditure of ATP, GTP and amino acids during translation.

Some examples included in this invention which concern the principle of a continuous flow of the feeding solution via the reaction volume (CFCF) are aimed at lowering the cost of preparative synthesis of target polypeptides. The feeding solution flow and concentrations of selected components can be readily controlled by changing the rate and direction of the flow

with a pump. Other examples demonstrate the use of the continuous exchange (CECF) and the possibility to increase productivity of the synthesis due to continuous changes in the concentration of at least one of the selected components. As known, the rate of exchange of low molecular weight components included in the reaction mixture and in the feeding solution via the dialysis membrane depends on many conditions (membrane area, pore size, etc.). This restricts the choice of modes of synthesis and the choice of upper and lower limits of the range for changing the concentrations of selected components. For example, in a CECF mode, it is more preferable to perform synthesis in separate modes (transcription, translation, transcription-translation) or in a combination of two modes (e.g. transcription and transcription-translation or transcription-trasnlation and translation), or in a combination of three modes (e.g. transcription, transcription-translation and translation). This is caused by the fact that due a rather low rate, the exchange takes a lot of time and cannot correspond to the rate required for input of low molecular weight components necessary for the synthesis and removal of low molecular weight products inhibiting the operation of the system.

Figure 1 shows two examples of changes of Mg^{2+} concentrations in the reaction mixture for different modes of synthesis. One example demonstrates the case when it is necessary to carry out synthesis of a large amount of mRNA. As seen from the diagram (K), changes of concentrations of selected components from the upper level (A) to level (C) are in the range within which conditions of predominant transcription and mRNA synthesis are formed in the reaction mixture due to high concentrations of Mg^{2+} and NTP. Here the initial concentration of Mg^{2+} should not exceed that of NTP by more than 10 mM. The following decrease in the concentration of Mg^{2+} and NTP is caused by the fact that the system adjusts Mg^{2+} and NTP concentrations to the corresponding values of the upper limit of the transcription-translation zone (C-D zone). The other example (diagram L) demonstrates the case when the upper limit of Mg^{2+} and NTP concentrations corresponds to the upper limit of the transcription-translation zone (C-D zone) and the lower limit coincides with the lower limit of the translation zone (D-B zone). In this case, conditions of the reaction mixture change from predominantly transcriptional to predominantly translational during the synthesis. Parameters of the porous barrier (pore size, membrane area, membrane type) and the rate of the feeding solution flow over the surface of the porous barrier should be chosen with account for the diffusion rate and exchange of low molecular weight components of the feeding solution and the reaction mixture, so that they would provide the required exchange rate and changing of the Mg^{2+} and NTP concentrations during the synthesis. The choice of the upper and lower limits of the concentrations depends on the properties of the cell-free extract that can be prepared in

different ways. The properties of the reaction mixture depend on the percentage of the extract and feeding solution contained in the mixture. Determination of the upper and lower limits, within which concentrations of the selected components are changed during the synthesis, allows to control the productivity of the cell-free system in different modes.

The continuous flow mode (CFCF) allows a rapid change of the rate and direction of the feeding solution flow via the reaction mixture. This permits to control the rate of changing the concentrations of selected components at various stages of the synthesis. During one run it is possible to choose different rates of the feeding solution flow via the reaction mixture. Therefore it becomes possible, during synthesis of polypeptides in a transcription-translation cell-free system, to adjust duration of individual stages at which parameters of the reaction mixture and concentrations of selected components correspond to those of predominant transcription, transcription-translation or translation. The choice of definite parameters within which concentrations of selected components can be changed depends on the aim of synthesis (synthesis of mRNA, or synthesis of a target polypeptide in the translation mode, or synthesis of a target polypeptide in the transcription-translation mode), selected conditions of synthesis and, first of all, properties of the cell extract, parameters of porous barriers (pore size, membrane area and membrane type), possibility to add expendable high molecular weight components. The upper limit of the allowable range of Mg^{2+} concentrations (from which a working range is determined) cannot exceed 50 mM in CFCF transcription-translation systems with DNA-dependent RNA polymerase. The lower limit of Mg^{2+} concentrations cannot be below 0.25 mM.

Figure 2 shows the dependence (M) of changes in Mg^{2+} concentrations on the time of transcription-translation synthesis. Adjusting the rate of the feeding solution flow at the first stage of synthesis (t_1-t_2 period) it is possible to correct the amount of synthesized mRNA and prevent their overproduction. The high concentration of Mg^{2+} and NTP at the beginning of the first period (t_1-t_2) decreases the required amount of expensive RNA polymerase, because mRNA synthesis proceeds with a lower yield of abortive mRNA. The ratio of Mg^{2+} and NTP concentrations is selected so that at the first stage Mg^{2+} concentration exceeds NTP concentration by a value of 5 to 10 mM, whereas at the third stage this excess of Mg^{2+} over NTP would not be less than 0,5 mM.

Upon a prolonged synthesis in a CFCF mode, concentrations of selected components are changed from the upper to the lower limit once or recurrently. Figure 3 shows a diagram (N) of changes in Mg^{2+} and NTP concentrations at a recurrent pulse input of the additional mixture to the reaction mixture. The synthesis is divided in N steps with a step duration from t_4

to t_6. The additional mixture is introduced during the t_4-t_5 time. Mg^{2+} and NTP concentrations increase, pass level C, and conditions of synthesis in the reaction system achieve zone A-C where predominant transcription of mRNA takes place. A decrease in Mg^{2+} and NTP concentrations changes conditions of synthesis from transcription-translation (zone C-D) to predominant translation (zone D-B).

During preparative synthesis of target polypeptides not only low molecular weight components of the feeding solution are required for the prolonged synthesis. In this mode in addition to low molecular weight components, high molecular weight components are also introduced into the reaction mixture. These components are (I) a ribosomal fraction, (ii) a cell-free extract (S30, S100 and their modifications), (iii) polymerases, (iv) plasmids, (v) tRNA. With regard to the conditions of synthesis, high molecular weight components are introduced once, recurrently or continuously. It is preferable to introduce such components as polymerases and plasmids to the reaction mixture together with the input of the maximal concentration of Mg^{2+} and NTP at the stage of transcription. The ribosomal fraction may be input upon translation.

Figure 4 shows the diagram (O) of changes in Mg^{2+} and NTP concentrations upon formation of linear gradient of these components. The process of linear gradient formation is well known and widely used, e.g., in liquid chromatography. It is advisable to use this mode for a preparative synthesis of the target polypeptide in the system of mRNA translation. In this mode, Mg^{2+} and NTP concentrations, which are in the range of changes (zone E-F), should correspond to the range in which Mg^{2+} and NTP concentrations are most close to the optimal translation of mRNA. The range of allowable Mg^{2+} and NTP concentrations should be determined from the known types of extracts given in the literature or from technical descriptions of manufacturers. A decrease in the efficiency of translation in zones close to the limits of the E-F range is compensated by multiple iterations of the synthesis conditions via the optimum zone with a recurrent change in Mg^{2+} and NTP concentrations proportional to the linear gradient shape. As in the above case, the whole synthesis is divided in N steps with each step duration varying from t_7 to t_9. At the first step, t_7-t_8, the additional mixture containing high Mg^{2+} and NTP concentrations is mixed with the feeding solution, so that Mg^{2+} and NTP concentrations in the total mixture increase. The total mixture is introduced to the reaction mixture and changes conditions of the synthesis. At the time, it maintains the synthesis and removes from the reaction volume low molecular weight components inhibiting the synthesis. With a change in the ratio of the mixed volumes and a decrease of input amount of the additional mixture relative to the feeding solution, Mg^{2+} and NTP concentrations in the total mixture decrease. The decreased Mg^{2+} and NTP concentrations pass the region of the E-F

zone where the synthesis is maximal. Depending on conditions of the synthesis, excess high molecular weight components maintaining the synthesis are added to the reaction volume continuously or recurrently.

A similar mode can be used also for controlling the preparative transcription in order to obtain a sufficient amount of mRNA. The difference of the known methods for synthesis of mRNA in a batch mode and the methods with fed batch of transcription systems without removal of low molecular products (Kern and Davis, 1997) is as follows. (a) Due to removal of low molecular weight components inhibiting synthesis of mRNA, the process of synthesis is prolonged and the yield of mRNA increases. (b) Due to the choice of the lower limit of the range for Mg^{2+} and NTP concentrations, it is possible to obtain mRNA in conditions that promote the next stage of translation of the synthesized mRNA without additional purification. (c) The use of high Mg^{2+} concentrations (up to 50 mM) results in a decreased yield of abortive or incomplete mRNA molecules and lowers the consumption of expensive RNA polymerase.

4. Reactor module

The above modes of operation can be realized by a proper choice of a design of the reaction module. Using porous barriers which are placed inside the reaction module, a reaction volume is formed as well as zones for both input of the feeding solution and additional mixture components, expendable high molecular weight components maintaining the synthesis, and output from the reaction module of low molecular weight components inhibiting the synthesis, and in some modes output of high molecular weight components including the target polypeptides.

In the simplest construction, the reaction volume is divided into two zones: (a) in a CECF mode, the volume is divided by a porous barrier in the zone with the reaction mixture and the zone with the feeding solution; (b) in a CFCF mode, the volume is divided by a porous barrier in the zone with the reaction mixture where the feeding solution is added, and the zone for removal of synthesized products (US Patent 5478730, Alakhov et al., 1995).

There are reactors in which three zones are formed (US Patent 5135853, Dzewulski et al., 1992; US Patent 5478730, Alakhov et al., 1995; DE Patent Appl. 19832160.0 A1, Bauer et al., 1999): a zone for the feeding solution input, a zone with the reaction mixture and a zone for output of sythesized products. Such a division is required for maintaining constant conditions of synthesis. In this invention, the productivity of synthesis should be supported, on the one hand, by a constant composition of amino acids and other components in the reaction mixture and, on the other hand, by active (in the CFCF mode) or passive (in the CECF mode)

regulation of concentrations of selected components. These conditions determine the choice of a construction of reactors designed for operation in different modes. The construction of a reactor with two porous barriers forming three zones in the reactor volume is most widely used in different modes. The number of zones can be increased as required by the peculiarities of the reactor module.

The reaction mixture volume depends on conditions and purposes of synthesis. As known (US Patent 5324637, Thompson et al., 1994), for research purposes the synthesis is performed in microvolumes. Synthesis on a preparative scale (European Patent 0593757, Baranov et al., 1997) is performed in reactors whose volume varies from 500 µl to 1.0 ml. For research purposes, the minimal reaction volume should be from 50 to 500 µl. For preparative synthesis, one or several reaction modules with a volume from 500 µl to 10 ml are used. The number of reaction volumes in the reactor may vary from 1 to 10 depending on the types of reactor modules.

Three processes should proceed simultaneously in each point of the reaction volume: (a) input of the feeding solution, (2) output of low molecular weight products inhibiting the synthesis, and (3) a temporary change in the concentration of selected components determining the productivity of the synthesis. The most preferable is the reactor module in which various shapes of thin layers of the reaction mixture are formed. The layer thickness is chosen so that the continuous exchange of the reaction mixture and the feeding solution components or the feeding solution flow via the reaction mixture, as well as removal of low molecular weight products, inhibiting the synthesis, would proceed during the period when the synthesis does not drop below the allowable level. The reaction mixture can be placed to the volume of any shape formed between the surfaces of the porous barriers. With the use of hollow fibers, flat membranes or their combinations, the reaction volume can have the form of a cylinder or a thin flat sheet of 0.1 to 5.0 mm thickness. The internal volumes of the reaction system and the feeding solution can intermix either due to a closed loop circulation of the reaction mixture using a pump (US Patent 54343079, Mozayeni, 1995), due to shaking the reactor (US Patent 5593856, Choi, 1997) or due to the use of a magnetic stirrer (Kim and Choi, 1996). The reaction volume can be previously filled with different separators or extenders of an organic or inorganic nature. They can be of porous, layered or capillary materials chosen from the following: (a) filters from synthetic polymers or inorganic materials, (b) porous metals or their composition, (c) gel-like structures. Porous materials with pore sizes from 10 µm to 0.1 mm placed in the reaction mixture serve to increase the area on which molecules collide with each other and thus increase the rate of the synthesis reactions (Alberts

et al., 1983). In addition to polymers, inorganic oxides and ceolytes (US Patent 5593856, Choi, 1997), these materials may comprise sorbents used in chromatography, including affinity sorbents (Maier et al., 1998) to isolate the target polypeptide from the reaction system. The use of any porous material for this purpose is restricted only by their chemical activity and possibility to inhibit the synthesis.

Porous barriers such as membranes, hollow fibers and other porous structures, ensure exchange of the feeding solution and reaction mixture components and play the role of distributors of the feeding solution flows via the reaction volume. There are no restrictions concerning the use in one reactor of porous barriers of different types (membranes, hollow fibers) and of different materials (solid or solid in a combination with gel). Porous barriers can be used both as one-layer or multi-layer constructs including those made of various materials.

The proposed herein variants of mutual positions of porous barriers can be modified using the existing knowledge of the art.

EXAMPLES OF REALIZATION OF THE INVENTION

Below are given examples of flow formation (the feeding solution, additional mixture or their combination) in reactor modules for an efficient synthesis in the continuous exchange (CECF) or continuous flow (CFCF) modes (example 1 - example 5) and of synthesis of chloramphenicol acetyl transferase (CAT) in continuous exchange mode (example 6).

Example 1

Fig. 5 shows a scheme of reactor 10 with one porous barrier 11 which divides the reactor volume into two parts. In one part 14 restricted by the surface of porous barrier 11, is placed the reaction mixture introduced via inlet 1. In the other part 15, via inlet 2 the feeding solution is introduced which comes in contact with the surface of porous barrier 11. The porous barrier can have the form of a flat sheet or a cylinder. In the first case, dialysis or ultrafiltration membranes in the form of a disk, a square or a rectangle are used; in the second case, hollow fibers or dialysis containers. The reaction mixture is incubated at a temperature from 20 to 40°C. The temperature range preferable for wheat germ extract is 20-26°C, for reticulocyte lysate it is 24-38°C, and for *E.coli* extract it is 20-38°C. Tangential flows which move along the internal and external surfaces of the membrane are formed to intensify the exchange of the feeding solution and the reaction mixture. For the CECF mode, such porous barriers are chosen which would allow removal of only low molecular weight components from the reaction volume (when pore sizes do not exceed 30 kD) or simultaneous removal of low and high

molecular weight components (when pore sizes vary from 30 to 100 kD). Inlets 1 and 2 of the reactor module can be closed hermetically or open during synthesis with the same pressure being maintained in both parts of the reaction volume. This permits to add substrates, which maintain the synthesis, to the reaction mixture or to the feeding solution and also to change the concentration of selected components independently of the diffusion. Before the synthesis, the volume ratio of the reaction mixture and the feeding solution is chosen to be from 1/5 to 1/100, and types of the reaction module are selected to correspond to this ratio, pore size and area of the dialysis membrane.

Description of the use of the proposed method for synthesis in the continuous exchange mode (CFCF) is given below.

For analytical purposes, synthesis of polypeptides is performed in microreactors with a reaction volume no smaller than 50 μl. Synthesis of preparative amounts of polypeptides imposes special requirements to the mode of synthesis and design of the reactor. Below are given variants which can be realized with a one-channel and multichannel reactors including those with branched flows inside the reactor volume. Variants of porous barriers, their parameters and thickness of the reaction mixture layer are mostly analogous to those described above.Reactors used in a flow mode should ensure (1) input of the feeding solution containing low molecular weight components to the reaction volume and (2) input of high molecular weight components directly to the reaction volume or via a porous barrier. The porous barrier plays the role of a distributor of flows with pore sizes not exceeding 5000 kD which ensure free penetration of most components of the S30 extract, excluding ribosomes and complexes formed around it. The number of possible constructions of reactors that can be designed using the existing knowledge is rather great, and the prior art does not restrict the range of other variants.

Example 2.

Synthesis in the mode of a continuous input of the feeding solution with branched flows of output fractions of high and low molecular weight components (CFCF-BF) provides a possibility to concentrate the synthesized polypeptide inside the reaction mixture due to independent regulation of output flows. Figure 6 shows a scheme of the reactor and directions of flows formed at a branched output of high molecular weight fraction F40 and low molecular weight fraction F30. The reactor has a housing 10, two porous barriers 11 and 12 which form reaction volume 14 located between internal surfaces of porous barriers, and two zones 15 and 16 for input/output of liquid circuits which contact the external surface of the porous barriers. The reaction mixture is input to the reaction mixture via inlet 1. To the same inlet are

supplied (a) feeding mixture F10, (b) additional mixture F20, (c) fraction of high molecular weight components F50. The feeding mixture is supplied to inlet 1 continuously or recurrently. Depending on conditions of synthesis, additional mixture F20 and fraction of high molecular weight components F50 are supplied to the reaction mixture once, recurrently or continuously. Fraction of high molecular weight components F50 is input to the reaction volume independently of feeding solution F10, or high molecular weight components are preliminarily mixed with the feeding solution. Synthesis may be performed without input of fraction F50. Prior to its beginning, ratios of the volumes of fractions of the feeding solution, the additional mixture and the high molecular weight fraction to those of the reaction mixture and the flow rate of these fractions via the reaction volume are determined. Pore sizes of the first porous barrier 11 are selected, taking into account the molecular weight and dimensions of the target polypeptide, in the range from 30 to 100 kD; pore sizes of the second porous barrier 12 are taken not to exceed 30 kD. With regard to the selected mode of synthesis, the ratio of volumes passing via the first and second barriers is taken to be from 1/10 to 1/100.

Example 3

Figure 7 shows a scheme of flows of a reactor where the first and second barriers have the same or different pore size not exceeding 30 kD. In this case, synthesis proceeds without removal of high molecular weight fraction F40 and the target product from the zone of synthesis (CFCF-RP), and flows F31 and F32 contain only low molecular weight components. This mode is used when the synthesized product exceeds 80-100 kD or when accumulation of the synthesized polypeptide in the reaction volume does not inhibit the synthesis. Modes of input of the feeding solution, the additional mixture and the fraction with high molecular weight components are similar to the CFCF-BF mode.

Example 4

Porous barriers can be used as distributors of the feeding solution and additional mixture flows when the volume, to which the reaction mixture is introduced, is filled with porous materials and mixing of the reaction volume is impaired or impossible.

Figure 8 shows a scheme of the reactor module and the flow direction in a CFCF-RP mode when the first porous barrier plays the role of a distributor of flows of feeding solution F10 and additional mixture F20. Pore sizes of the first porous barrier 11 should not exceed 5000 kD. This permits to input part of the high molecular weight fraction F51 via the first porous barrier, if dimensions of the components included in this fraction are smaller than the pore size of the first barrier. Such components are tRNA, enzymes and others. When required, ribosomal fraction F52 is input to reaction volume 14 directly via liquid inlet 1. Pore sizes of the

second porous barrier should not exceed 30 kD. The flow of low molecular weight components F30, inhibiting the system operation, is removed via outlet 5.

Example 5

Figure 9 represents a scheme of flows for the CFCF-RF mode when the direction of the feeding solution supply via the first and second porous barriers is recurrently altered. In this mode designated for a prolonged synthesis of polypeptides, alteration of the direction of the feeding solution supply provides clearing the pores of the first 11 and second 12 porous barriers. The synthesis is performed either without removal of high molecular weight products from the reactor (pore sizes of the first and second barriers are taken to be the same and do not exceed 30 kD), or with removal of part of the synthesized product from the reaction volume (pore size of the first porous barrier should not exceed 100 kD, and that of the second porous barrier should not exceed 30 kD). The flow of high molecular weight components F50 is supplied to the reaction volume via inlet 1. N steps of the input of the feeding solution and the additional mixture are formed during synthesis. Each step is divided into two periods. During the first period, containers with the feeding solution and the additional mixture are connected to inlet 2 using liquid valves. The flows of the feeding solution F11 and the additional mixture F21 are input to zone 16 formed by the surface of the first porous barrier 11. Via pores of the first porous barrier, the feeding solution and the additional mixtures are supplied to the zone of synthesis 14 of the reactor. Via pores of the second porous barrier 12 low molecular weight components are removed from the zone of synthesis to zone 15, and then flow 32 is formed which is removed from the reactor via outlet 5. After termination of the first period, the valves are switched and containers with the feeding solution and the additional mixture are connected to inlet 3 which is linked to zone 15 formed by the second porous barrier 12. Flows F12, F22 penetrate via the second porous barrier to the reaction mixture and at the same time clear the pores of the second barrier which have closed during the first step of the synthesis. Low molecular weight components leave the reaction volume via the first porous barrier. They form flow F31 which is removed from the reactor via outlet 4. By adjusting the duration of the first and second periods for the input of the feeding solution and additional mixture flows to the reaction volume via the first and second porous barriers, the volume ratio of flows F31 and F32 is changed.

Example 6.

Synthesis of chloramphenicol acetyl transferase (CAT) in the continuous exchange mode (CECF) in the translation system and in two variants of the combined transcription-translation systems.

It is known that transcription of a circular and linearized form of DNAs by phage polymerases proceeds in different conditions, in particular, at different Mg^{2+} concentrations. Linear DNA templates can include plasmids linearized by restriction enzymes and PCR products. The use of PCR products excludes involvement of living cells for preparing genetic constructs (e.g., upon expression of genes coding for unstable or stable toxic products (Martemyanov et al., 1997)). Moreover, the use of PCR for preparing templates provides for an easier and more convenient modification of their constructs at the genetic level, including (a) introduction of elements stabilizing the RNA structure (e.g., highly structured regions, RQ elements of RNA, terminators of transcription etc.), (b) introduction of elements enhancing gene expression (e.g., enhancers, non-translatable leaders etc.), (c) introduction of coding marker sequences (e.g., epitopes, TAG for affinity isolation etc.).

The method provides for alterations in the intensity of transcription both from the circular plasmids that can be, for some reasons, in a supercoiled form and from plasmids having the form of a relaxed ring. For different polymerases, such forms are inherent to templates whose efficiency depends on Mg^{2+}.

Below is given an example of application of this method when circular plasmids are used upon CAT synthesis in the transcription-translation system.

To compare the productivity of synthesis in different modes, several reaction mixtures were used. The first reaction mixture was used to prepare the translation mixture, and CAT was synthesized using the earlier prepared mRNA. In the second mode, components for mRNA transcription were added to the reaction mixture and conditions were created for the combined transcription-translation. In the third variant, an additional mixture consisting of selected components, such as Mg^{2+} and NTP, was added to the reaction mixture prepared for synthesis in the combined transcription-translation system. Additional components were introduced to the reaction mixture before the synthesis.

a) Synthesis of CAT in a cell-free translation system

The reaction mixture for mRNA translation was prepared taking into account the data given in Table 1. The mixture included a wheat germ extract.

Table 1. Composition of the reaction mixture for translation

Components	Final concentration in the reaction mixture
Wheat germ extract	30% v/v
CAT enhancer mRNA	100 µg/ml
Yeast tRNA	0.005 mg/ml
RNAse inhibitor	133 U/ml
Protease inhibitor cocktail (1)	
25-fold dilution	1 X
19 amino acids (each)	0.1 mM
[^{14} C] Leucine, 38 mCi/mmol	0.1 mM
ATP	1 mM
GTP	0.8 mM
Creatine-phosphate	10 mM
HEPES-KOH pH 8.0 (2)	53 mM
KOAc (2)	100 mM
$Mg(OAc)_2$ (2)	4.2 mM
DTT (2)	1.3 mM
Spermidine	0.1 mM
β-mercaptoethanol	2 mM
Glycerol	4%
H_20	to the final volume

(1) Protease inhibitor cocktail "Complete", Boehringer Mannheim GmbH.

(2) Final concentrations take into account the contribution of concentrations of $Mg(OAc)_2$, KOAc, DTT, HEPES introduced by the wheat germ extract (Boehringer Mannheim GmbH).

The feeding solution was prepared according to the data given in Table 2.

Table 2. Composition of the feeding solution for translation

Components	Final concentration in the feeding solution
HEPES-KOH pH 8.0	53 mM
KOAc	100 mM
$Mg(OAc)_2$	4.2 mM
DTT	1.3 mM
β-mercaptoethanol	2.0 mM
Spermidine	0.1 mM
ATP	1.0 mM
GTP	0.8 mM
Creatine-phosphate	10 mM
Glycerol	4%
[^{14} C] Leucine, 38 mCi/mmol	0.1 mM
19 amino acids (each)	0.1 mM
H_20	to the final volume

A dialyser prepared from a dialysis container of 8 mm in diameter (Union Carbide Corp.) and with the operation volume of 100 µl was used in this example. The volume of the feeding solution was 1 ml. To compare the productivity of the synthesis, the total reaction mixture was divided into two volumes. 30 µl of the reaction mixture were placed in a microcentrifuge tube, and 100 µl of the mixture were placed in the dialyser. The dialyser and the microtube were placed in a thermostated volume and synthesis was performed at 25°C. During the synthesis, 5 µl aliquots were taken from the microtube and the dialysis container to determine the kinetics of synthesis in the batch static mode and in the continuous exchange (CECF) mode. The amount of the synthesized polypeptide was determined by precipitation of the synthesized polypeptide on the glass fiber filter with trichloroacetic acid followed by radioactive counting in a liquid scintillation counter. Figure 10 shows the kinetics of the synthesis. Diagram P refers to the synthesis in a batch mode. Diagram R demonstrates the kinetics of CAT synthesis upon translation in the CECF mode.

b) CAT synthesis in the combined transcription-translation system

The reaction mixture was prepared in accordance with the data given in Table 3. The combined transcription-translation system contains the plasmid of pCAT ennancer with the gene of chloramphenicol acetyl transferase (CAT) under the SP6 polymerase promoter.

Table 3. Composition of the reaction mixture for the combined transcription-translation

Components	Final concentration in the reaction mixture
Wheat germ extract	30% v/v
pCAT-enhancer plasmid	50 μg/ml
SP6 RNA polymerase	15000 U/ml
Yeast tRNA	0.005 mg/ml
RNAse inhibitor	133 U/ml
Protease inhibitor cocktail (1)	
25-fold dilution	1 X
19 amino acids (each)	0.1 mM
[^{14} C] Leucine, 38 mCi/mmol	0.1 mM
CTP	0.4 mM
UTP	0.4 mM
ATP	1 mM
GTP	0.8 mM
Creatine-phosphate	10 mM
HEPES-KOH pH 8.0 (2)	53 mM
KOAc (2)	100 mM
$Mg(OAc)_2$ (2)	5.0 mM
DTT (2)	1.3 mM
Spermidine	0.1 mM
β-mercaptoethanol	2 mM
Glycerol	4%
H_20	to the final volume

(1) and (2) are the same as in Table 1.

The feeding solution was prepared taking into account the data given in Table 4. To maintain the transcription process, CTP and UTP were added to the feeding solution.

Table 4. Composition of the feeding solution for transcription-translation

Content	Final concentration in the feeding solution
HEPES-KOH pH 8.0	53 mM
KOAc	100 mM
$Mg(OAc)_2$	5.0 mM
DTT	1.3 mM
β-mercaptoethanol	2.0 mM
Spermidine	0.1 mM
ATP	1.0 mM
GTP	0.8 mM
CTP	0.4 mM
UTP	0.4 mM
Creatine-phosphate	10 mM
Glycerol	4%
[^{14} C] Leucine, 38 mCi/mmol	0.1 mM
19 amino acids (each)	0.1 mM
H_20	to the final concentration

Conditions of the synthesis (temperature, reaction mixture volume, feeding solution volume, type of dialyser) were the same as those in Example 6a (CAT synthesis in the translation system). The results of the synthesis were analyzed as in Example 6a.

Figure 11 shows the kinetics of CAT synthesis in the combined transcription-translation system. Diagram S represents synthesis in the batch mode. Diagram T represents the kinetics of CAT synthesis upon transcription-translation in the CECF mode.

c) CAT synthesis in the combined transcription-translation system at a continuous change of Mg^{2+} and NTP concentrations in the reaction mixture during synthesis

The reaction mixture was prepared taking into account the data given in Table 5.

Table 5. Composition of the reaction mixture for the combined transcription-translation at a continuous change of Mg^{2+} and NTP concentrations during synthesis

Components	Final concentration in the reaction mixture
Wheat germ extract	30% v/v
pCAT-enhancer plasmid	50 μg/ml
SP6 RNA polymerase	15000 U/ml
Yeast tRNA	0.005 mg/ml
RNAse inhibitor	133 U/ml
Protease inhibitor cocktail (1)	
25-fold dilution	1 X
19 amino acids (each)	0.1 mM
[^{14} C] Leucine, 38 mCi/mmol	0.1 mM
CTP	0.8 mM
UTP	0.8 mM
ATP	2.0 mM
GTP	1.6 mM
Creatine-phosphate	10 mM
HEPES-KOH pH 8.0 (2)	53 mM
KOAc (2)	100 mM
$Mg(OAc)_2$ (2)	11.2 mM
DTT (2)	1.3 mM
Spermidine	0.1 mM
β-mercaptoethanol	2 mM
Glycerol	4%
H_20	to the final volume

(1), (2) are the same as in Table 1

The feeding solution is prepared with account for the data given in Table 6.

Table 6. Composition of the feeding solution for the combined transcription-translation at a continuous change of Mg^{2+} and NTP concentrations during synthesis

Components	Final concentration in the feeding solution
HEPES-KOH pH 8.0	53 mM
KOAc	100 mM
$Mg(OAc)_2$ (1)	3.8 mM
DTT	1.3 mM
β-mercaptoethanol	2.0 mM
Spermidine	0.1 mM
ATP	1.0 mM
GTP	0.8 mM
CTP	0.4 mM
UTP	0.4 mM
Creatine-phosphate	10 mM
Glycerol	4%
[^{14} C] Leucine, 38 mCi/mmol	0.1 mM
19 amino acids (each)	0.1 mM
H_20	to the final volume

The concentration of $Mg(OAc)_2$ in the feeding solution was reduced to 3.8 mM, since during the synthesis when the concentrations of the reaction mixture and the feeding solution become the same, the concentration of $Mg(OAc)_2$ raises to 5.1 mM which is appropriate to transcription-translation.

Conditions of the synthesis (temperature, volume of the reaction mixture and that of the feeding solution, type of dialyser) were selected analogous to those given in Example 6a (CAT synthesis upon translation). The results of the synthesis were analyzed as described in Example 6a.

Figure 12 shows the kinetics of CAT synthesis in the combined transcription-translation system. Diagram U represents synthesis in the batch mode. Diagram V represents the kinetics

of CAT synthesis upon transcription-translation in the CECF mode with changing concentrations of the Mg^{2+} and NTP in the reaction mixture during the synthesis.

Figure 13 shows a diagram which compares the results of experiments on the synthesis of the target CAT polypeptide. The data are taken from examples 6a-c and demonstrate the yield of CAT (μg/ml) in different modes: (a) static (batch) mode of combined transcription-translation (example 6b, bar W); (b) translation (example 6a, bar X); (c) combined transcription-translation (example 6b, bar Y); (d) combined transcription-translation with changing concentrations of Mg^{2+} and NTP in the reaction mixture (example 6c, bar Z). A comparison of the results shows that the highest yield of CAT polypeptide (32 μg/ml) is obtained in the combined transcription-translation mode when concentrations of Mg^{2+} and NTP are changed from their maximal to minimal values.

INDUSTRIAL APPLICABILITY

The invention can be used to synthesize polypeptides in cell-free systems using eukaryotic and prokaryotic cells. The method described herein allows the user to optimize the entire synthesis and to study the contribution of individual components to the synthesis at separate modes of transcription, transcription-translation and translation.

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WHAT IS CLAIMED IS:

1. A method for obtaining polypeptides in a cell-free system by which the reaction mixture is prepared with the use of a cell lysate or cell extract, the parameters of the cell-free system and the mode of synthesis are chosen, the type and parameters of at least one porous barrier are determined, the reaction mixture and the feeding solution are placed in the reaction module, and the synthesis is performed, ***wherein*** upon the parameters of the process are chosen, the types of the selected components determining the productivity of the synthesis are selected, the upper and lower limits of the range within which the concentrations of the selected components are changed during the synthesis are defined, the additional mixture containing the selected components is formed, the additional mixture is supplied to the reaction mixture or to the feeding solution, the synthesis is performed with changing concentrations of the selected components within the defined ranges while the concentrations of the other components are maintained constant.

2. The method according to claim 1 ***wherein*** at least one of the selected components is chosen from the group consisting of Mg^{2+}, K^{+}, NTP, polyamine or their combination.

3. The method according to claim 2 ***wherein*** one combination of the selected components includes Mg^{2+} and NTP.

4. The method according to claim 1 ***wherein*** the mode of synthesis is chosen at least from one mode selected from a group consisting of translation, transcription-translation, transcription or combinations of these modes.

5. The method according to claim 4 ***wherein*** depending on the mode of synthesis, NTPs contained in the additional mixture consist of a group of ATP, GTP, UTP and CTP or a group of ATP and GTP.

6. The method according to claim 1 ***wherein*** the additional mixture is supplied to the reaction mixture before the synthesis or during the synthesis, or the additional mixture is supplied to a part of the feeding solution before the synthesis or during the synthesis.

7. The method according to claim 6 ***wherein*** the additional mixture is supplied once, recurrently or continuously during the synthesis.

8. The method according to claim 1 ***wherein*** the mode of input of low molecular weight components of the feeding solution to the reaction mixture is selected from a group of continuous exchange modes, a group of continuous flow modes, or a combination of these modes.

9. The method according to claim 1 ***wherein*** the reaction mixture is prepared using a cell lysate or cell extract obtained from prokaryotic or eukaryotic cells.

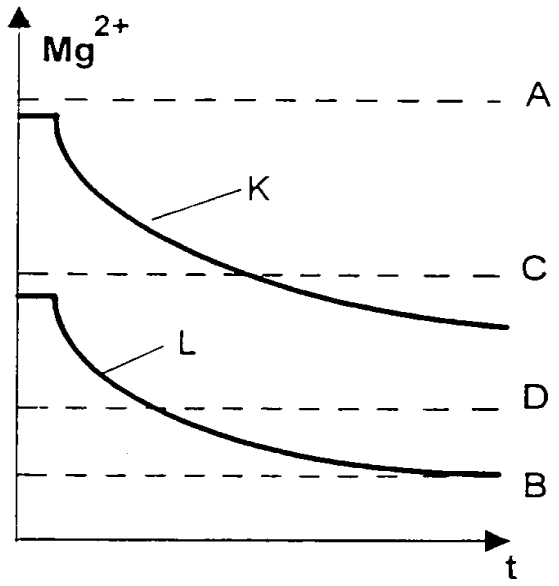


Fig. 1

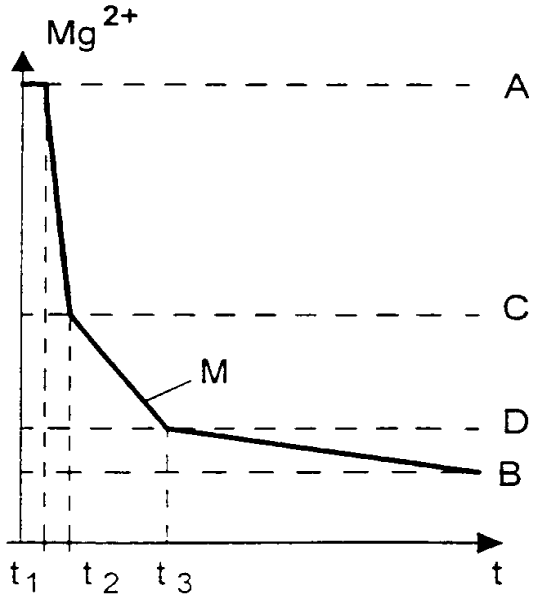


Fig. 2

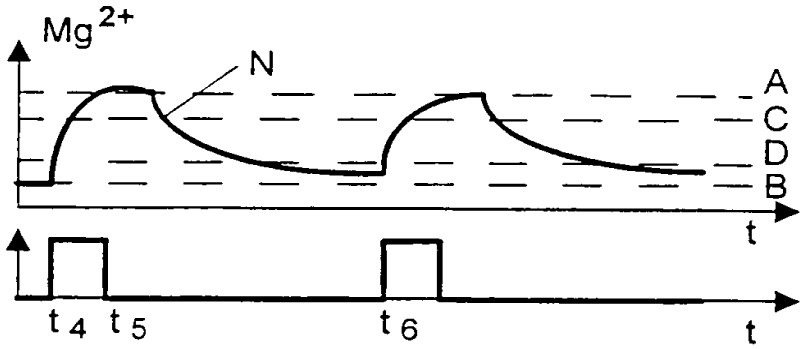


Fig. 3

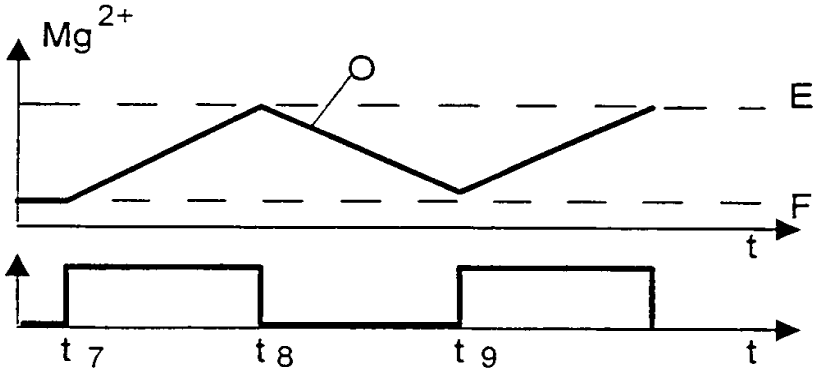
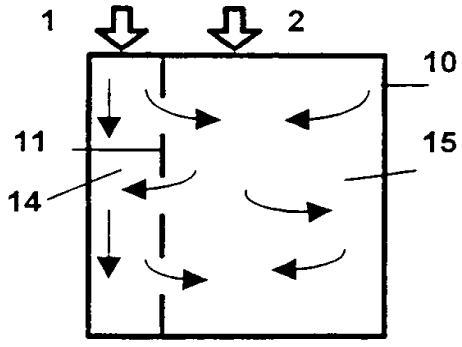


Fig. 4



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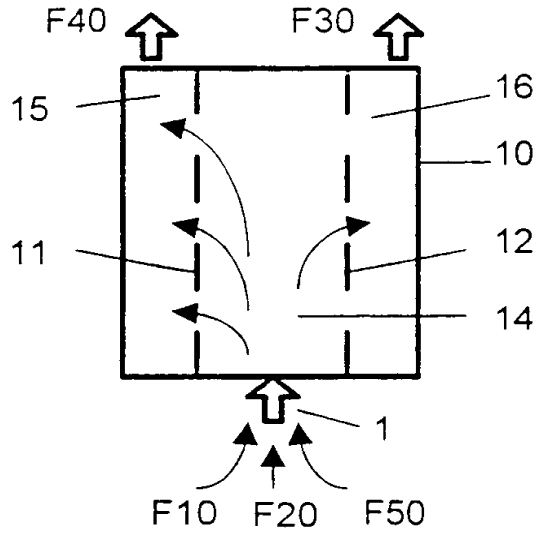

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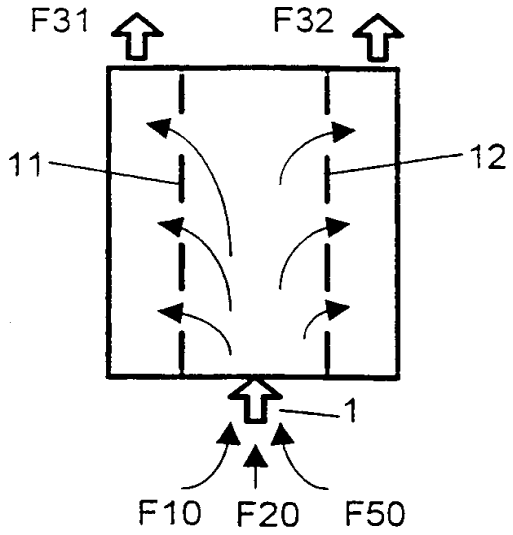

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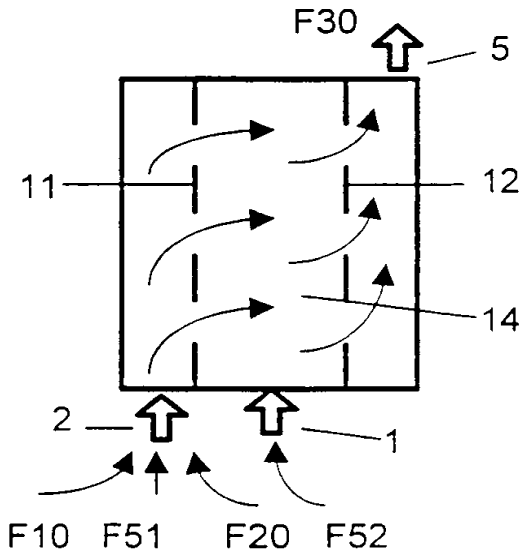


Fig. 8

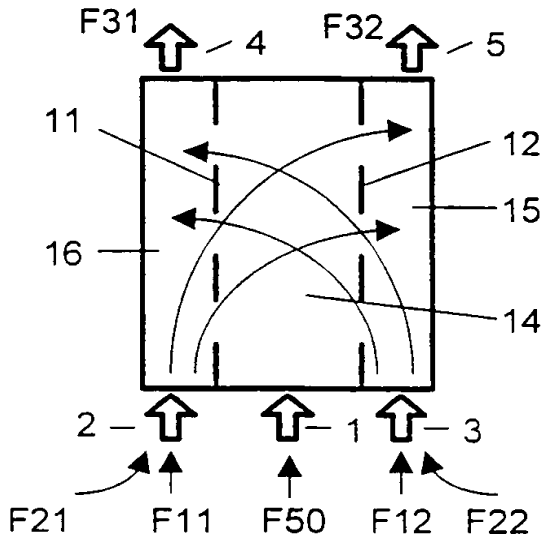
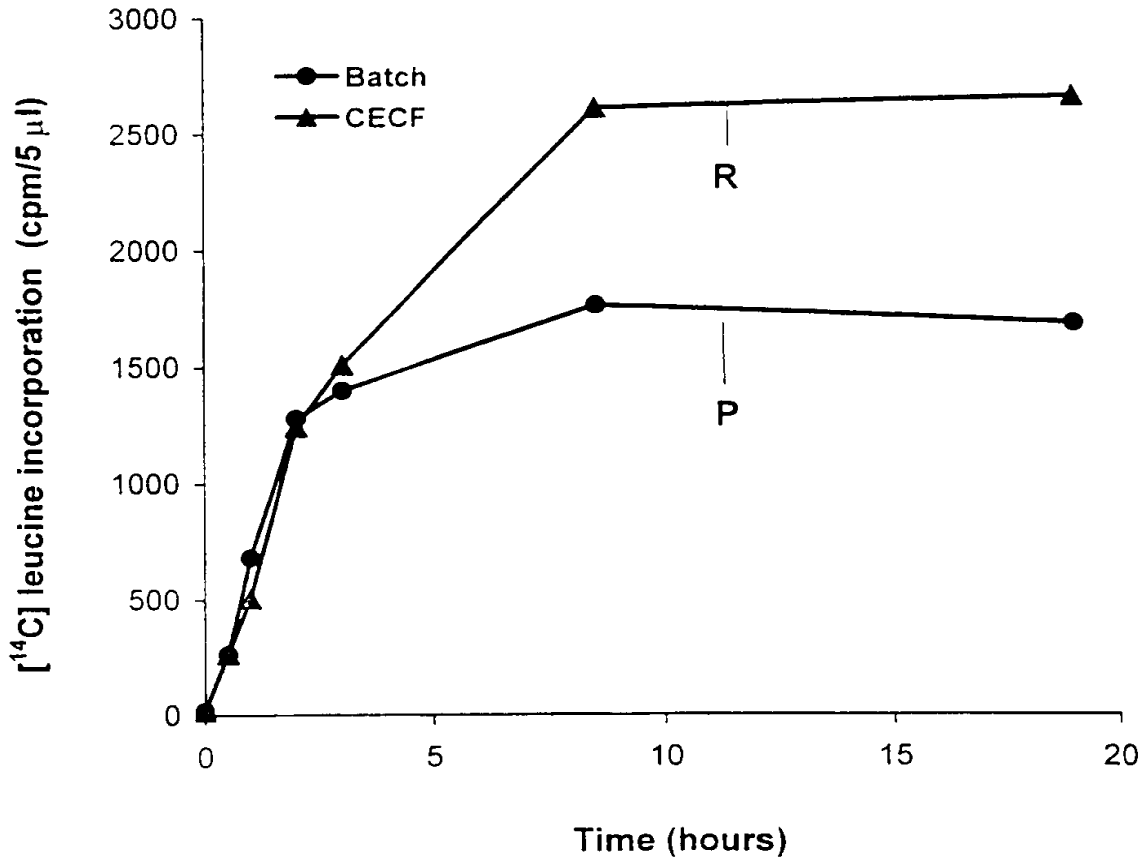


Fig. 9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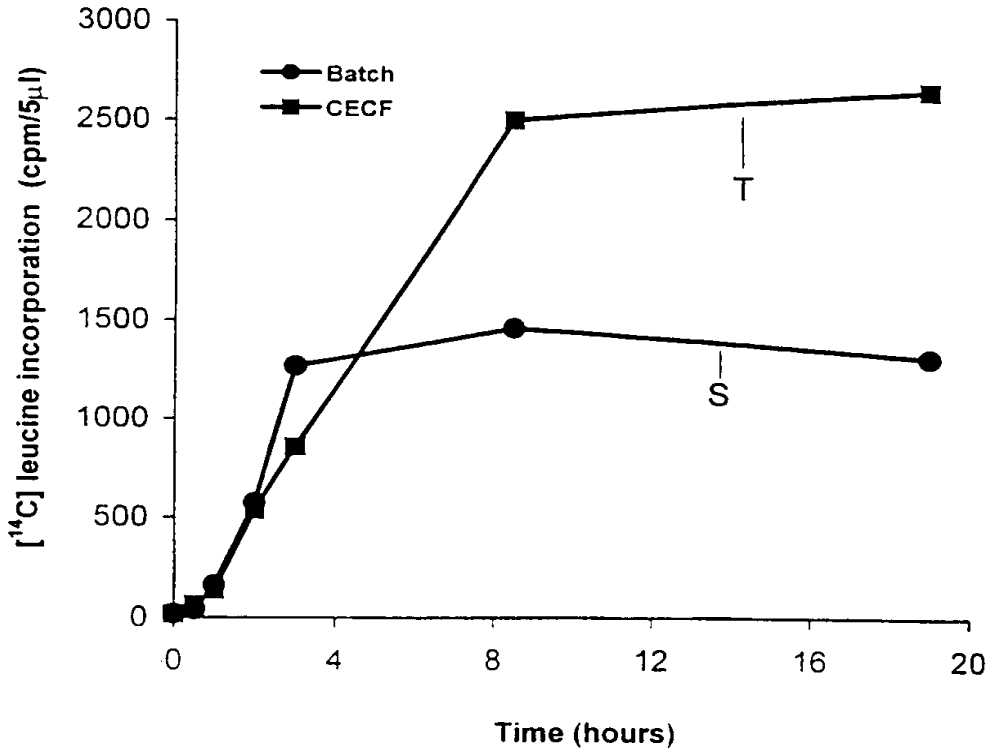
1500
1000
500
0
0
5
10
15
20
Batch
CECF
R
P
[14C] leucine incorporation (cpm/5 µl)
Time (hours)

Fig. 10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4
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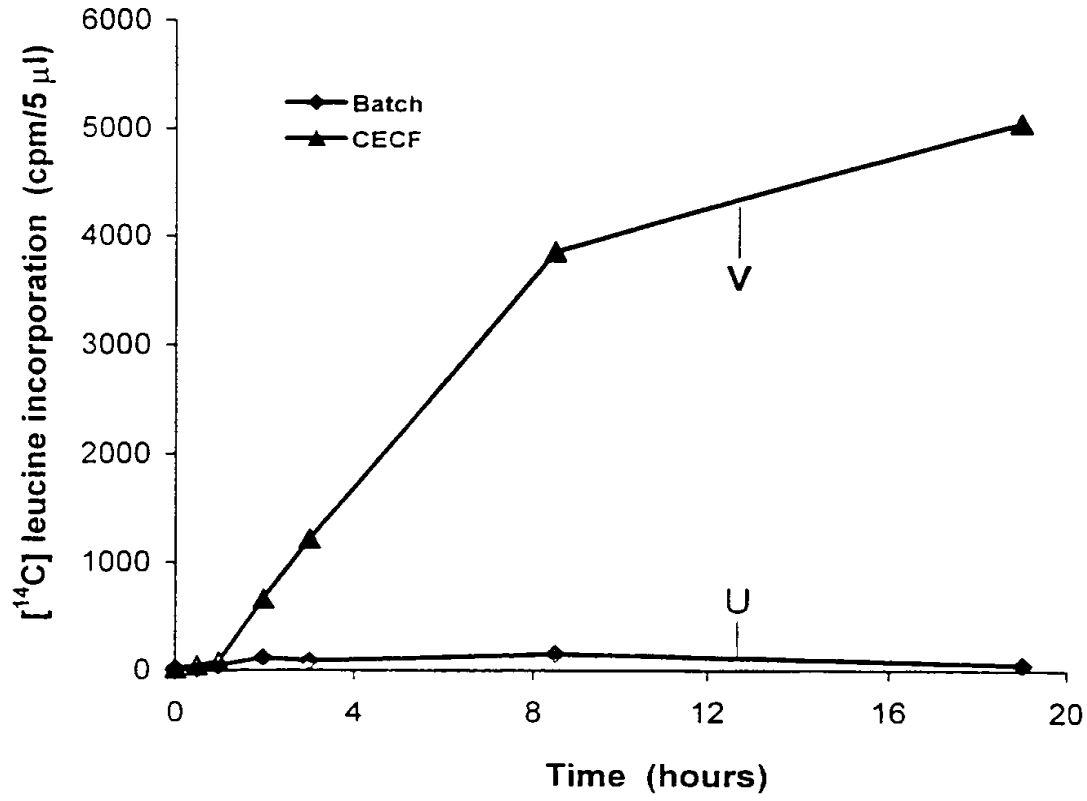
12
16
20
Time (hours)

Fig. 11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Batch
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V
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8
12
16
20
Time (hours)

Fig. 12

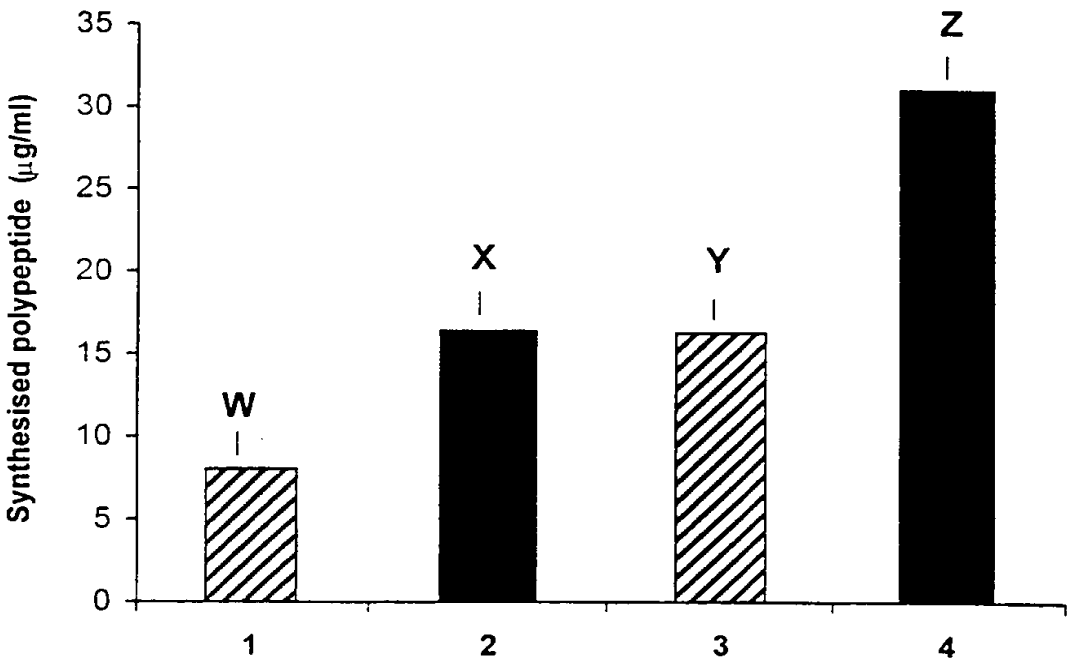

Fig. 13